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INFLUENCE OF pH AND TEMPERATURE ON FERULIC ACID ESTERASE AND ACETIC ACID ESTERASE ACTIVITIES DURING MALTING AND MASHING

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The influence of malting temperature (according to "Activated Germination Malting") and decreased pH of steeping water on malt ferulic acid esterase activity and acetic acid esterase activity were studied. The pH value of water applied for steeping did not influence ferulic acid esterase activity changes during malting, and an increase of the malting temperature to 22°C caused a decrease in ferulic acid esterase activity in kilned malt. Ferulic acid esterase was not thermostable and a decrease in ferulic acid esterase activity was observed at 35°C. In order to promote ferulic acid esterase activity during mashing, the temperature of the mash must be held below 40°C during the first stages of the mashing process. Acetic acid esterase is another accessory enzyme taking part in arabinoxylan degradation during malting and mashing. Application of the water with a decreased pH level (5.2 *versus* 7.4) during steeping of the grain of both tested barley varieties caused nearly complete acetic acid esterase inactivation during malting. During mashing, acetic acid esterase was inactivated at 45°C, and the role of this enzyme during mashing is considered to be marginal.

INTRODUCTION

Ferulic acid esterase and acetic acid esterase are accessory enzymes taking part in the degradation of arabinoxylans during malting. Ferulic acid esterase shows the ability to release free ferulic acid which forms ester bonds with arabinoxylan backbone chains. The enzyme activity is present in barley and increases during steeping. The highest ferulic acid esterase activity is present after 2 days of germination followed by a decrease in enzyme activity during the next days of the process. The enzyme activity is present in the aleurone layer and in the endosperm, but ferulic acid esterase activity in the aleurone layer of the grain is 10–20 fold higher than in the endosperm. The enzyme is active towards all the methyl esters of the cinnamic acid derivatives except methyl caffeate, but the highest activity of the enzyme was observed when methyl esters of p-coumaric or ferulic acid were used. Barley ferulic acid esterase is very active towards a natural feruloylated trisaccharide composed of an arabinofuranose and two xylose units (*FA-Af-Xylp-Xylp*) [Sancho *et al*., 1999]. Higher activity towards the natural substrate than the synthetic one could suggest a higher affinity of the enzyme for the arabinoxylan chain fragments present in cell walls. Barley ferulic acid esterase exhibits no activity towards methyl esters of the benzoic acid derivatives, but additionally, the enzyme exhibits acetic acid esterase activity [Bartolome *et al*., 1996]. Purification and separation of the enzymes responsible for ferulic acid esterase activity in barley allowed isolation of a number of proteins of molecular masses in the range of 22 kDa – 158 kDa, but the highest ferulic acid esterase activity was recorded in the case of protein of molecular mass about 138 kDa [Humberstone & Briggs, 2002b]. Incubation of a malt extract, possessing "ferulic acid esterase" activity, with barley spent grain resulted in the release of small concentrations of ferulic acid. What is more important, in the presence of a xylanase produced by *Trichoderma viridae,* the release of ferulic acid was about 10-fold higher. Application of the xylanase from *Trichoderma viridae* alone, with no "ferulic acid esterase activity", did not cause the release of ferulic acid, which proves the existence of cooperation between these two enzymes during the degradation of arabinoxylans. The optimal pH value for the action of malt "ferulic acid esterase" was 7.5. Because the pH value in the endosperm of germinating barley grain is 4.5-5.5, "ferulic acid esterase" cannot act at its optimal pH value [Humberstone & Briggs, 2000b].

Another enzyme important during the degradation of non-starch polysaccharides is acetic acid esterase. β-1,4-xylopyranose units in arabinoxylan chains can be esterified at the O-2 or O-3 position with acetic acid residues. Acetic acid residues form *ca*. 2.1% of the cell wall mass, and ferulic acid residues are equal to *ca*. 1.2% of the mass of the cell walls of barley grain. Taking into consideration the molar masses of the two compounds, it can be concluded that the degree of esterification of arabinoxylan chains with acetic acid in barley cell walls is much higher than esterification by ferulic acid. Acetylation of the arabinoxylan chains by acetic acid causes the formation of a double helical structure of arabinoxylan chains in solution. Acetylation is also responsible for better solubility of arabinoxylans in solution, whereas removal of acetic acid residues from arabinoxylans can cause the formation of arabinoxylan aggregates. The optimal pH value for acetic acid esterase activity is 7.0 and the molecular mass of the enzyme isolated from barley is 55 kDa [Humberstone & Briggs, 2002a]. Strong cooperation of acetic acid esterase and endo-1,4-β-xylanase produced by *Trichoderma reesei* was reported by other authors, because in the absence of endo-

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1,4-β-xylanase, acetic acid esterase activity was decreased [Biely *et al*., 1986]. The aim of this study was to evaluate the changes of ferulic acid and acetic acid esterase activities during malting and mashing, as well as to determine the influence of a decreased pH value of steeping water and elevated temperature during malting on the two enzymes' activities.

MATERIALS AND METHODS

Materials and reagents. Two barley varieties were used: Krona (Semundo Saatzucht GmbH) and Rudzik (Hodowla Roślin Szelejewo). The samples were gathered in the year 1999. Barley malt for lager-type beer and hops granulate (4.2%$_{w/w}$ of α-acids) were obtained from Perła Browary Lubelskie S.A. P-nitrophenylacetate (for acetic acid esterase activity determinations) and methyl esters of phenolic acids (for ferulic acid esterase activity determinations) were purchased from Apin Chemicals Ltd, Oxon, UK.

TECHNOLOGICAL OPERATIONS

Malting on a laboratory scale. Pale malts for lager beer were produced. Malting conditions on a laboratory scale were comparable to those described by Nischwitz *et al*. [1999]. A portion of 1 kg of barley grain was malted in a vertical glass vessel (2 L). The total volume of water in the system was 4.5 L. The temperature was controlled with water. The time-temperature programme was as follows: 0–1 h – steeping the grain with simultaneous disinfection with 0.5 mL/L of hydrogen peroxide solution; 1–24 h – steeping the grain in water at pH 7.4 or pH 5.2; 24–34 h – air rest of the grain, the process was carried out in a chamber equipped with air flow and temperature control; 36–48 h – steeping the grain in water; 48 h – germination. Germination was continued until the proper length of rootlets was achieved [Kunze, 1999]. Therefore the total malting times of particular malts are different. Malt heating was performed in 4 successive steps: 10 h at 40°C; 10 h at 55°C; 4 h at 72°C; 4 h at 83°C. In order to investigate the influence of elevated temperature on the activities of barley grain enzymes [Baca *et al*., 1998a,b], malting was performed at 22°C, as described by Kitamura *et al*. [1990]. To study the influence of decreased pH value of steeping water on barley enzyme activities, two pH values were also applied: pH 7.4 (tap water) and pH 5.2 (tap water with lactic acid (150 g/L of solution)). Samples of malts were withdrawn every 24 h during steeping and germination and after every stage of kilning. Directly after kilning, grain rootlets were removed by rubbing through a strainer. Malt had been stored for 4 weeks at 4°C before the analyses were undertaken. Malting experiments were repeated in duplicate for each of the two barley varieties tested and for each malting technology applied (temp. 14°C or 22°C and pH of steeping water 7.4 or 5.2).

Wort production. All worts were produced using the infusion method, according to Kunze [1999]. Malt for lager-type beer was ground on a laboratory scale in such a way that intact husks and flour (finely milled endosperm) were obtained. Wort was produced in a Braun fermentor (B. Braun Biotech International, Type C10-3, working volume of 15 L), with the controlling unit Braun Biostat C Type 884492/5. Tap water (pH 7.4) was applied for the wort production, and the pH value was decreased and continuously controlled at 5.2 with 20% lactic acid solution. Cooling of the worts was performed with the use of tap water. During mashing, the wort was continuously mixed (at 150 rpm). The mashing programme comprised the following stages: 2.4 kg of ground malt was mixed in the fermentor with water (10 L, 40°C); next the pH level of the medium was adjusted to 5.2 and the mixture was then warmed at: 52°C (20 min), 63°C (20 min), 75°C (20 min). Sweet wort was then filtered at 75°C using barley spent grain (husks) as a filtration medium following removal of the barley spent grain from the fermentor and filling the fermentor with a sweet wort and, finally, boiling the wort with hops (2.011 g of hops granulate, 4.2% of the α-acid content) for 90 min. at 101°C.

ANALYTICAL METHODS

Preparation of enzyme extracts from barley and malt. Acetic acid esterase and ferulic acid esterase extractions from barley and malt samples were performed according to Humberstone & Briggs [2000a,b, respectively]. Barley grain (15 g) was ground in a laboratory mill and extracted in conical flasks using an extraction buffer (100 mL) of the following composition: Tris/HCl (50 mmol/L, pH 5.5 for acetic acid esterase and pH 8.0 for ferulic acid esterase); reduced glutathione (GSH) (25 mmol/L); Triton-X-100 (1 g/100 mL); 1% PVPP (1 g/100 mL). Extraction flasks were closed with stoppers and put at ambient temperature in a shaker (165 rpm) for 3 h. After this, a part of the extract (usually 30 mL) was purified from the solid parts by centrifugation (5000 g, 20 min). After centrifugation, the volumes of the extracts were checked and adjusted to the initial ones using Tris/HCl buffer. Samples of malts withdrawn at different stages of the malting process were characterised by different water contents, and it was not possible to separate equal samples from every stage of the malting by weight. Because of that, use was made of the method proposed by Sancho *et al*. [1999]. Taking into consideration the mass of 1000 barley kernels, the number of kernels from every stage of malt production equal to 15 g was counted and extraction was performed as described above. For ferulic acid esterase activity determination, dialysis was not necessary. Before acetic acid esterase activity determination, dialysis of the extract was performed. Dialysis tubes (Sigma-Aldrich, 10 kDa cut-off) were filled with the extract (20 mL) and dipped in Tris/HCl buffer (0.05 mol/L, pH 5.5). The buffer was changed every 12 h. After dialysis, the volume of the extracts was adjusted to the initial one using Tris/HCl buffer (50 mmol/L, pH 5.5). Extracts were stored in a refrigerator and used within 1 week. The results of the ferulic acid esterase and acetic acid esterase activity determinations are referred to the volume of the enzyme extract.

Determination of ferulic acid esterase activity. Ferulic acid esterase activity was evaluated using 4 substrates: methyl ferulate, methyl coumarate, methyl vanillate and methyl syringate. The method proposed by Sancho *et al*. [1999] was applied. The solution of the substrate was prepared by solubilisation of the methyl ester of phenolic acid in 0.5 mL of pure methanol following addition of 9.5 mL of the buffer solution previously used for enzyme extraction (50 mmol/L of Tris/HCl, pH 8.0); the concentration of the substrate was 8 mmol/L. Next, 350 μL of the substrate solution and the

enzyme extract (or wort sample, 1 mL) were put into an Eppendorf tube. Samples were incubated for 5 h at 30°C. Then the enzyme was inactivated in boiling water for 5 min. and the samples were then cooled and centrifuged (30 min, 5000 g). Simultaneously, samples with inactivated ferulic acid esterase (5 min, in boiling water) prior to the addition of the substrate were incubated. After incubation, free phenolic acids – ferulic, coumaric, vanillic or syringic acid – were separated from their corresponding methyl esters using HPLC with UV detection according to the method of McCrae *et al*. [1994]. Chromatographic separations were performed in an isocratic system. The eluent was water:buthanol:acetic acid 93.8:6.0:0.2. The HPLC system consisted of: Rheodyne loop (20 μL), a KNAUER mini piston pump, UV/Vis detector LINEAR 200 (USA) and a TZ 4620 recorder (Czech Republic). Column Symmetry® C_{18} (Waters, 250 mm, 4.6 mm i.d., 5 μm) was used for separations. The flow rate was 0.8 mL/min. Detection of ferulic acid and coumaric acid was performed at 320 nm, and that of vanillic and syringic acid – at 280 nm. The eluent was prepared in distilled water and degassed in an ultrasonic bath before use. Concentrations of free phenolic acids were determined by using the heights of the chromatogram peaks. The series of methanol solutions of phenolic acids were prepared and the calibration curves were constructed taking into consideration the heights of the peaks and the corresponding concentrations. Ferulic acid esterase activity was always calculated on the basis of free phenolic acid release in the sample after incubation with the enzyme. Enzyme activity is expressed as the free phenolic acid concentration released in 1 min of incubation and is referred to the volume of the enzyme extract (mmol/L/min) [Humberstone & Briggs, 2000b].

Determination of acetic acid esterase activity. Enzymatic activity was determined by using p-nitrophenyloacetate as a substrate according to the method of McCrae *et al*. [1994] with modifications. P-nitrophenyloacetate solution (0.01 g/100 mL) was prepared using a 96% solution of ethanol. Enzyme activities were determined after 1 h of incubation at 25°C. The composition of the reaction mixture was as follows: 0.1 mL of the malt extract or wort, 10 mL of 0.01 mol/L Tris buffer (pH 7.0), 0.5 mL of p-nitrophenyloacetate solution. Double blank samples were prepared: **I**. 0.1 mL of malt extract or studied wort, 0.5 mL of ethanol and 10 mL of 0.01 mol/L Tris buffer (pH 7.0); **II.** 10.1 mL of 0.01 mol/L Tris buffer (pH 7.0) and 0.5 mL of p-nitrophenyloacetate in an ethanol solution. Absorbances of incubated samples were read after 30 min. at 430 nm. The standard curve was prepared by dissolving 5 mg of p-nitrophenyloacetate in 5 mL of ethanol. From this solution, aliquots of 0.1 mL, 0.2 mL, 0.3 mL, 0.4 mL and 0.5 mL were made up to the volume of 10.6 mL using 0.1 mol/L NaOH solution. Acetic acid esterase activity was always calculated on the basis of the free p-nitrophenol concentration increase in the sample after incubation with the enzyme extract. Acetic acid esterase activity was expressed in activity units; one unit was the amount of the enzyme releasing 1 μmol of p-nitrophenol in 1 L of the reaction mixture during 1 min [McCrae *et al*., 1994].

Determination of ferulic acid esterase thermostability in malt extracts. Ferulic acid esterase thermostability determination was performed using methyl ferulate or methyl coumarate as the substrate and at two pH levels: at the optimal pH level for the action of the esterase (7.5) and at a pH level of 5.2, which is the pH recommended by Kunze [1999] for wort production. Malt enzyme extracts were heated for 1 h at 30–70°C, and the extracts were then cooled to 30°C following the remnant enzyme activity determinations.

Acetic acid esterase thermostability determination. Acetic acid esterase thermostability determination was performed at the pH level of 7.0. At the first stage, the enzyme extract was heated for 1 h in the temperature range 20–70°C. The extracts were then cooled to 20°C and the residual acetic acid esterase was evaluated as described earlier.

Extraction of free ferulic acid from wort samples. Extraction was performed according to the method of Pan *et al*. [1998]. Worts were centrifuged (30 min, 5000 g), then each wort sample (15 mL) was acidified to pH 2 using HCl solution (in distilled water) and extracted using 90 mL of ethyl ether. The extracts were then dried using Na_2SO_4, and the solvent was evaporated under vacuum. The residues were dissolved in HPLC grade methanol (3 mL) and the solutions were used for HPLC analyses.

Release of bound ferulic acid in wort samples by mild alkaline hydrolysis. Alkaline hydrolysis of the bound ferulic acid was performed according to the method of Pan *et al*. [1998] with slight modifications. Samples of worts were centrifuged (30 min, 5000 g), then 10 mL of 0.5 mol/L of NaOH solution was added to 5 mL of the wort sample. Samples were then placed for 24 h at ambient temperature (approx. 20°C) in darkness. After hydrolysis, the samples were acidified to pH 2 using HCl solution (in distilled water) and extracted using 90 mL of ethyl ether. The extracts were then dried by using Na_2SO_4, and the solvent was evaporated under vacuum. The residues were dissolved in HPLC grade methanol (5 mL) and the solutions were used for HPLC analyses.

HPLC analysis of free and bound ferulic acid contents in wort samples. HPLC in isocratic conditions, according to the method of Pussayanavin & Wetzel [1987], with modifications proposed by Zupfer *et al*. [1998], was applied. The mobile phase was methanol-citric acid buffer (sodium citrate-citric acid, 0.01 mol/L, pH 5.4; $13{:}87_{v/v}$). The HPLC unit consisted of: a KNAUER piston pump (Germany), a UV/VIS LINEAR 200 (USA) detector and a TZ 4620 recorder (Czech Republic). Symmetry® C_{18} (Waters, length: 250 mm, i.d. 4.6 mm, 5 μm) RP column was used for separations. The flow rate of mobile phase was 0.4 mL/min. Injection volume was 20 μL (Rheodyne loop); the detector was set at 320 nm. HPLC analyses were performed in triplicate and mean values were calculated. Eluent for chromatography was prepared in the distilled water and degassed in an ultrasonic bath.

RESULTS

Ferulic acid esterase activity in experimental malts

Ferulic acid esterase activity towards methyl ferulate in the Rudzik malt produced at 14°C (steeping and germination) and at a pH level of 7.4 increased from the second day

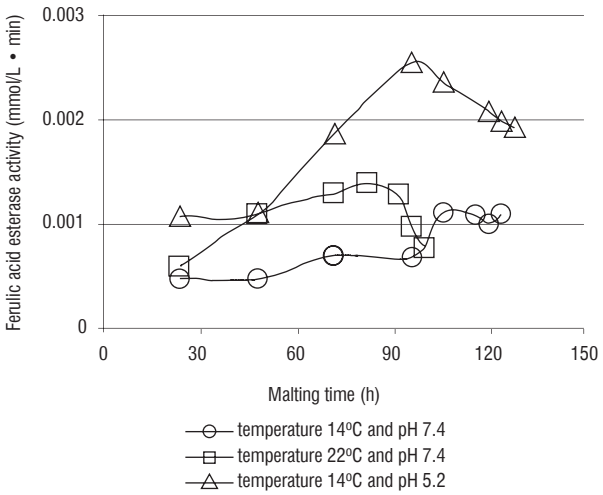

FIGURE 1. Ferulic acid esterase activity changes measured using methyl ferulate as a substrate in enzymatic extracts prepared from Rudzik malts.

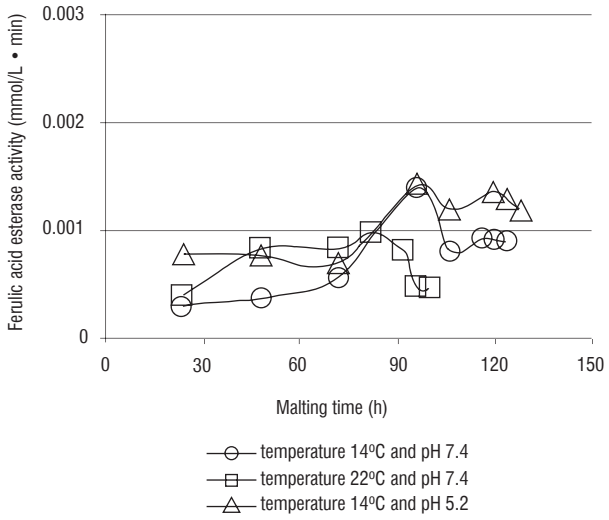

FIGURE 2. Ferulic acid esterase activity changes measured using methyl coumarate as a substrate in enzymatic extracts prepared from Rudzik malts.

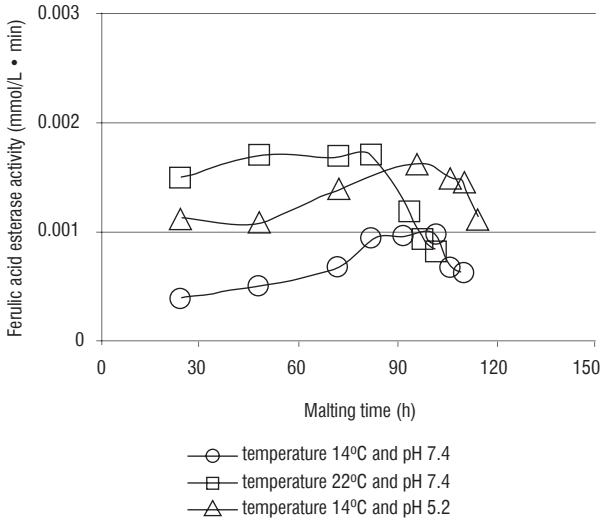

FIGURE 3. Ferulic acid esterase activity changes measured using methyl ferulate as a substrate in enzymatic extracts prepared from Krona malts.

of malting and did not significantly change until the end of the process (Figure 1). The activity of the enzyme measured towards methyl coumarate increased after 96 h of the process, but then decreased by approx. 36% during the first stage of kilning (Figure 2). The higher temperature during steeping (22°C) caused a more rapid increase in ferulic acid esterase activity in comparison to the malt produced at 14°C. Kilning at 55°C, 72°C and 83°C caused a significant decrease in ferulic acid esterase activity (Figures 1 and 2). This decrease reached approx. 45% and 52%, respectively, in comparison to the maximal enzyme activities determined after 82 h of the malting process. During steeping and germination of the Rudzik barley at 14°C and pH 5.2, an increase in ferulic acid esterase activity was observed in the first stages of the process. This was followed by a decrease in enzyme activity towards methyl esters of both phenolic acids (24% and 16% respectively, Figures 1 and 2), during kilning of the so-called "green malt" at 55°C, 72°C and 83°C. In general, ferulic acid esterase activity in extracts from malt produced at 14°C and a pH level of 7.4 was similar to enzyme activity in malt produced at the same steeping temperature but at a decreased pH level (5.2). Anyway, decreased ferulic acid esterase activity was determined in the malt produced at 22°C. The same experiment was repeated using a second barley variety.

Ferulic acid esterase activity in Krona malt produced at 14°C and pH 7.4 increased constantly until 102 h of the process. After kilning at 72°C, enzyme activity decreased by approx. 34% as compared to the maximal enzyme activity observed during the entire malting (Figure 3). Very similarly, ferulic acid esterase activity, studied using methyl coumarate as the substrate, increased until 106 h of the process, and then decreased (approx. 29%) during kilning at 83°C (Figure 4). Elevated temperature (22°C) during germination of Krona barley (Figures 3 and 4) caused an increase in enzyme activity in the early stages of malting. Maximal ferulic acid esterase activities were obtained after 82 h and 72 h of malting, when methyl ferulate or methyl coumarate were used as substrates, respectively. Kilning at 55°C, 72°C and 83°C caused a significant decrease in ferulic acid esterase activity towards both substrates. The decrease in enzyme activity, in comparison to the maximal enzyme activity, was 53% and 65% using

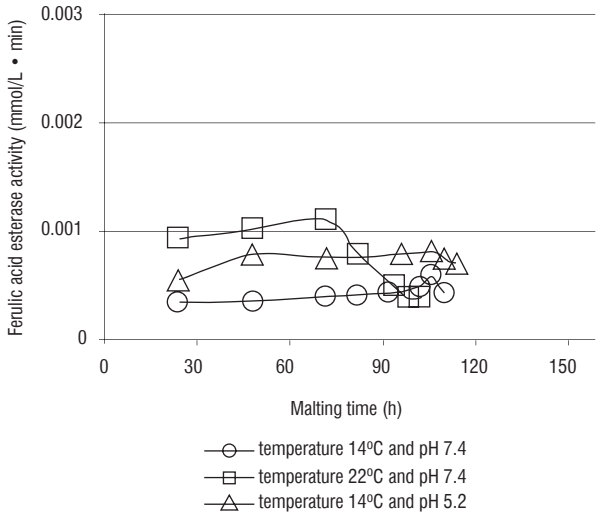

FIGURE 4. Ferulic acid esterase activity changes measured using methyl coumarate as a substrate in enzymatic extracts prepared from Krona malts.

methyl ferulate and methyl coumarate as substrates, respectively. Germination of Krona barley at 14°C and at a pH level of 5.2 caused an increase in ferulic acid esterase activity, with maximal activity after 96 h of malting, and after 106 h of the process, evaluated using methyl ferulate and methyl coumarate, respectively (Figure 4). Kilning of "green" Krona malt caused a decrease in enzyme activity towards both methyl esters of phenolic acids (approx. 31% and 14% using methyl ferulate and methyl coumarate as substrates, respectively). The highest ferulic acid esterase activity was obtained in the Krona malt produced at 14°C and pH 5.2. In summary, a significant decrease in ferulic acid esterase activity was observed in all experimental malts produced using two barley varieties. Because esterase is supposed to be present mainly in a mash which is a solution of much higher water content than malt grain, it was interesting to study the enzyme thermostability.

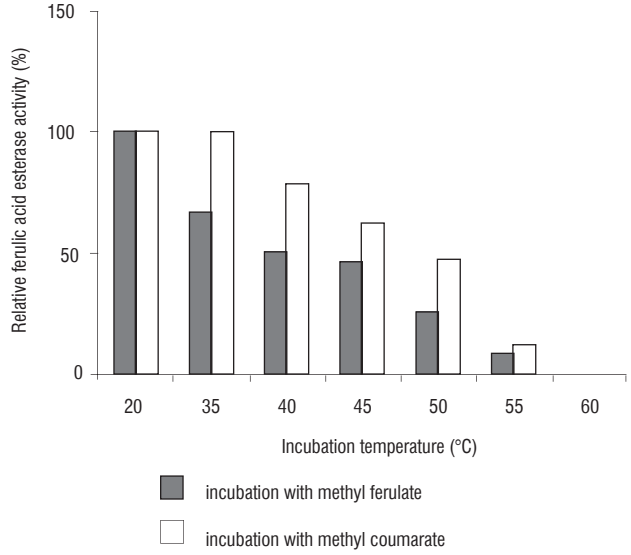

FIGURE 5. Ferulic acid esterase thermostability at pH 7.5 at different incubation temperatures.

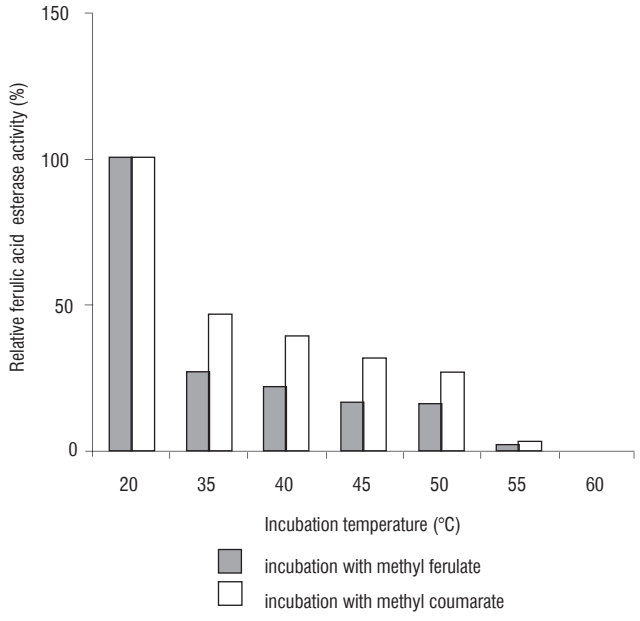

FIGURE 6. Ferulic acid esterase thermostability at pH 5.2 at different incubation temperatures.

Figure 5 presents the results of ferulic acid esterase thermostability studies performed at a pH level of 7.5. There was a significant decrease in enzyme activity at 35°C, and at 60°C ferulic acid esterase activity was completely inactivated. The enzyme showed higher thermostability when methyl coumarate and not methyl ferulate was applied as a substrate. The pH value during mashing is usually different from the optimal pH value for ferulic acid esterase activity, hence the thermostability of this enzyme at a pH level of 5.2 was evaluated as well (Figure 6). This pH value was chosen since Kunze [1999] pointed out that pH 5.2 was an optimal pH value for the promotion of many positive phenomena during mashing. The presented results prove a significant decrease in ferulic acid esterase activity at 35°C and total inactivation of enzyme activity at 60°C, similarly as observed in the case of studies performed at pH 7.5 (Figure 6). Figures 5 and 6 prove that pH value has a substantial significance for ferulic acid esterase activity during mashing and the non-heated enzyme showed more than 2-fold higher activity at pH 7.5 than at pH 5.2. In all studied barley and malt extracts, no ferulic acid esterase activity was detected, using methyl esters of benzoic acid derivatives as substrates.

Acetic acid esterase activity in malts

Figures 7 and 8 present the acetic acid esterase activity changes in two barley varieties during malting. Acetic acid esterase activities in malts of both varieties produced at 14°C and pH 7.4 increased after 48h of malting. In the case of Rudzik malt, enzyme activity was observed to decrease during kilning of "green malt", whereas in the case of Krona malt, it was reported to increase after the kilning. The elevated temperature during steeping and germination (22°C) while maintaining the pH value of steeping water at the level of 7.4 caused a significant decrease in acetic acid esterase activity in comparison to the malts produced at 14°C. The application of steeping water with decreased pH value (5.2 and not 7.4) caused a significant decrease in acetic acid esterase activity during each step of the malting process. Figure 9 presents the results of the acetic acid esterase activity determinations

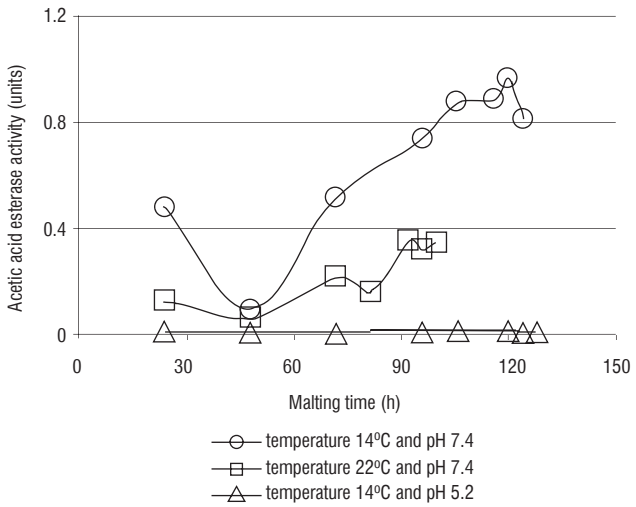

FIGURE 7. Acetic acid esterase activity changes in enzymatic extracts prepared from Rudzik malt.

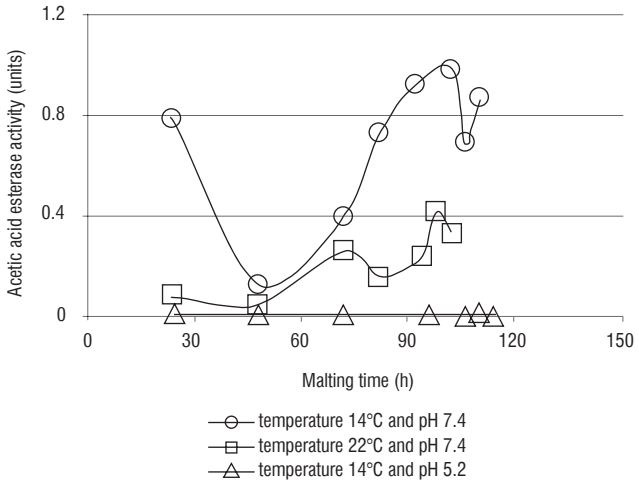

FIGURE 8. Acetic acid esterase activity changes in enzymatic extracts prepared from Krona malt.

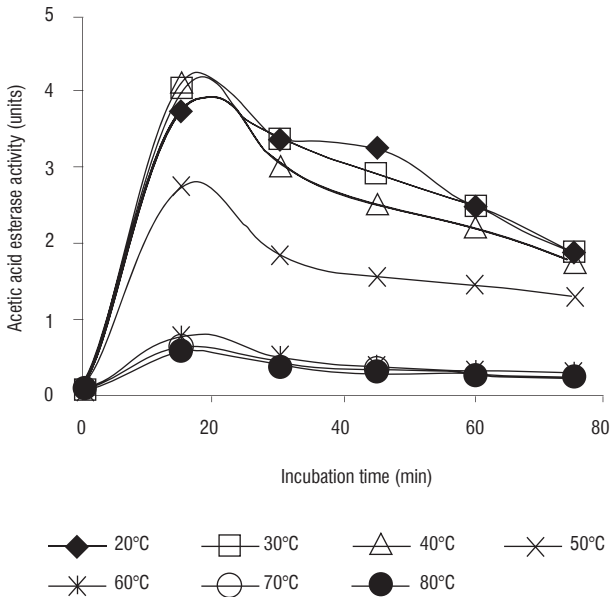

FIGURE 9. The influence of incubation time and temperature on acetic acid esterase activity.

at different temperatures. The heating of the malt extract at 60°C caused an approx. 8-fold decrease in enzyme activity in comparison to the non-heated enzyme.

Enzyme activities and ferulic acid concentration changes during mashing

In the next part of the experiments the study focused on an evaluation of the changes in ferulic acid esterase and acetic acid esterase activities during mashing. Free and total ferulic acid concentration changes during wort production were also studied. Figure 10 presents the time-temperature programme (a), malt ferulic acid esterase changes (b), malt acetic acid esterase activity changes (c), and changes of free and total ferulic acid concentrations during mashing (d). Malt ferulic acid esterase activity was significantly decreased at 52°C and the enzyme was inactivated at 63°C.

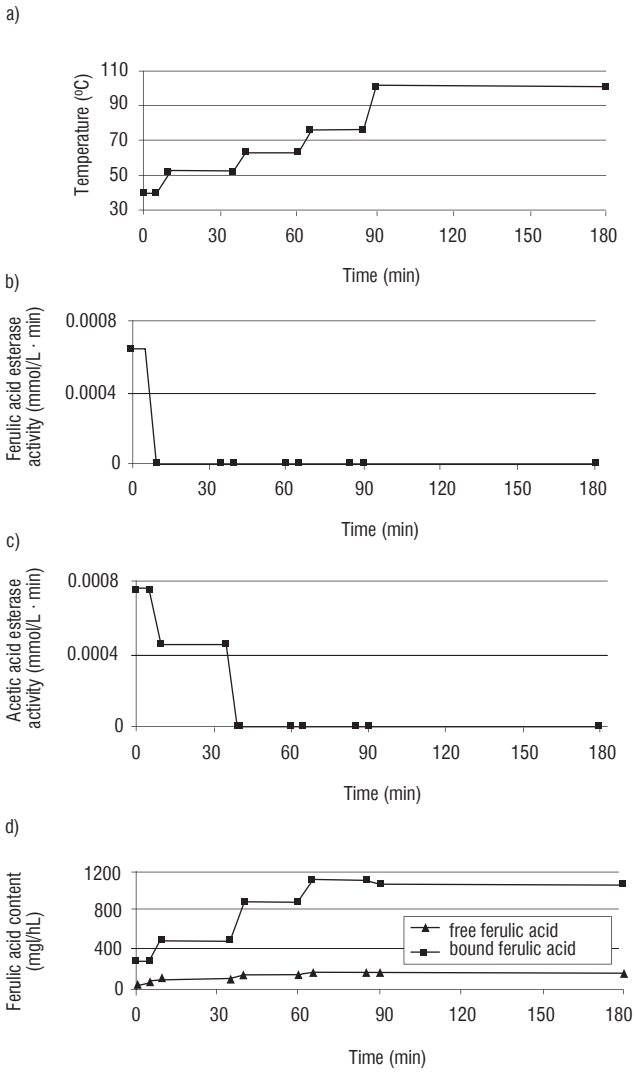

FIGURE 10. Enzyme activities and ferulic acid concentration changes during mashing. Time-temperature programme (a), ferulic acid esterase changes (b) acetic acid esterase activity changes (c), and changes of free and bound ferulic acid concentrations during mashing (d).

Acetic acid esterase activity was detected at 40°C during mixing of the malt grist with water, but at 52°C inactivation of the acetic acid esterase occurred. As for free and bound ferulic acid contents in wort, a continuous increase of both forms of ferulic acid contents was observed during mashing until wort boiling. After the break at 40°C, the concentration of bound ferulic acid reached 273.3 mg/100 L of wort and was approx. 5-fold higher than the free ferulic acid content, and during the next steps of mashing, the difference between the two forms of ferulic acid increased. After heating at 52°C, 63°C and 75°C, the ratio of bound ferulic acid to free ferulic acid content was 5.7, 6.5 and 6.9, respectively. After wort boiling, a decrease in bound ferulic acid and free ferulic acid content occurred, but the ratio of the two forms of ferulic acid remained unchanged and was approx. 6.8. It seems that the action of ferulic acid esterase causes the release of free ferulic acid to the solution and the concentration of bound ferulic acid should decrease, but in this experiment the bound ferulic acid concentration increased. This increase of bound ferulic acid concentration together with the increase in free ferulic

acid content during mashing can be explained by the simultaneous release of bound ferulic acid in a soluble form from insoluble fractions of non-starch polysaccharides present in solid parts of the malt grist.

DISCUSSION

Malting parameters, such as temperature and pH of the steeping water, can significantly influence the germination processes. Application of an elevated temperature of 22°C during malting was postulated by some researchers in order to replace the standard temperature of 14°C, as part of the malting technique referred to as "Activated Germination Malting" [Kitamura *et al*., 1990]. The elevated temperature of 22°C was intended to cause the activity of the main malt enzymes to appear at earlier stages of steeping of the grain, by fast activation of the embryo. "Activated Germination Malting" was intended to reduce the malting time without a loss of malt quality. Nischwitz *et al*. [1999] applied an elevated temperature of 22°C during steeping which resulted in decreased wort extract produced from the experimental malt, and decreased diastatic power of the malt. On the other hand, elevated temperature during steeping caused better β-glucans degradation, which resulted in decreased wort and beer viscosity, and in consequence allowed for higher filtration rates. Phenomena occurring during malting and mashing depend on the pH value and temperature, factors that also strongly influence ferulic acid and acetic acid esterase activities. The activity of ferulic acid esterase can be stimulated in order to increase the concentration of free ferulic acid, a strong antioxidant, in various beverages such as beer but also juices. The modern process of beer production is planned from the very beginning in a way that minimizes the oxygen uptake of beer, and therefore labile compounds, like ferulic acid, can be protected from oxidation. The desired, fresh flavour of beer can be saved although ferulic acid concentration in beer can be significantly increased. The course of changes of ferulic acid esterase and acetic acid esterase activity depends on the pH value and temperatures during the process. In particular, promotion of acetic acid esterase and ferulic acid esterase activities should be beneficial. Ferulic acid esterase activity increased during the first stages of malting, and a slight decrease in enzyme activity could be seen during kilning. This scheme of ferulic acid esterase activity changes was not dependent on the pH level of the steeping water or malting temperature and was similar in all experimental malts. In the present study, barley (malt) ferulic acid esterase exhibited high activity at a pH level of around 7.5, and at pH 5.2 (which is often applied during industrial mashing) ferulic acid esterase activity was strongly decreased. Humberstone & Briggs [2000b] evaluated remnant malt ferulic acid esterase activity towards ferulic acid glycerol esters after heating of the enzyme extract at 40°C. The authors suggested the presence of the soluble and insoluble form of ferulic acid esterase, because enzyme activity was detected in the enzyme extract as well as in insoluble fractions of cell walls isolated from malt. The results presented in this work and cited work show that ferulic acid esterase is not thermostable and its activity is decreased already at 45°C during mashing. Nevertheless, the present study shows that enzyme thermostability in the experimental worts was high and the optimal temperature for ferulic acid release during mashing was 45°C. The increased enzyme stability observed in this study could be the result of the higher concentrations of wort components like proteins and polypeptides, carbohydrates and others, which acted as protectors for enzymes. Ferulic acid esterase activity is present in barley, and the results of the present study are similar to the results obtained by other authors. Sancho *et al*. [1999] showed that the highest ferulic acid esterase activity was detected in grains after 2 days of germination, and during the next days of the process enzyme activity decreased. Ferulic acid esterase activity was present both in the aleurone layer and in the endosperm, but in the endosperm enzyme activity was 10-20 fold lower. The researchers recorded no ferulic acid esterase activity towards the esters of benzoic acid derivatives [Bartolome *et al*., 1996], and this result is in agreement with the results presented in this work (results not shown). Sancho *et al*. [1999] stated that ferulic acid esterase exhibited the highest activity towards methyl ferulate and ester of ferulic acid and two arabinofuranose residues (Fa-Ara*f*-Ara*f*) and no activity using methyl esters of benzoic acid derivatives as substrates. Also in the study presented in this paper, no ferulic acid esterase activity was detected in barley or malt extracts, using methyl esters of benzoic acid derivatives as substrates. It is very interesting that ferulic acid esterase activity is present in barley, whereas xylanase activity appears in barley (malt) grain after 5-6 days of the process [Sancho *et al*., 1999]. Other authors [Humberstone & Briggs, 2000b] recorded an increase in ferulic acid esterase activity towards ferulic acid glycerol ester during the first 3 days of malting. Steeping and germination was performed at 15°C, and kilning at 40°C for 2 days. The changes of ferulic acid esterase activity in the cited work was similar to the results obtained in the present work. Because of the difference in malting procedures in the two mentioned works, the similarity of the results could be attributed to the similar temperature during steeping and germination. The results of other studies [Bartolome & Garcia-Conesa, 1996] show that the highest ferulic acid esterase activity was obtained after 2 days of germination, but enzyme activity decreased during the following days.

Another important accessory enzyme during arabinoxylan degradation is acetic acid esterase. The pH of steeping water close to 5.2 during malting significantly reduced acetic acid esterase activity in comparison to the enzyme activity present during malting at a pH level of 7.4. Elevated temperature caused a decrease in enzyme activity in comparison to acetic acid esterase activity in malts produced at 14°C. Other authors [Humberstone & Briggs, 2000a] pointed out the acetic acid esterase inactivation at 40°C, with diacetin as a substrate. Malting performed with steeping water of pH 7.4 did not decrease acetic acid esterase activity, and the highest enzyme activity was obtained in the kilned malt. The above results prove that both temperature during mashing and pH of mash around 5.2 can cause complete inactivation of acetic acid esterase and the role of the enzyme during mashing can be marginal. Malt extract exhibited high acetic acid esterase thermostability during heating at 40°C, but elevated temperature of 50°C caused a significant decrease in enzyme activity. Other authors [Humberstone & Briggs, 2000a] pointed out the decrease in acetic acid esterase activity already at 40°C, but the substrate for the experiments in the cited work was diacetin. The authors of the cited work proved that barley

grain that underwent the malting procedure contained more than one acetic acid esterase, because there was no competition between the two substrates of the enzymes p-nitrophenylacetate and diacetin.

CONCLUSIONS

In conclusion, it can be stated that the highest ferulic acid esterase activity in malt was obtained while pH during steeping and germination was 5.2 and the temperature of the malting process was 14°C. The application of water of pH 7.4 suppressed ferulic acid esterase activity during steeping and germination of barley grain. By means of modified pH of steeping water and temperature of the malting process, it is possible to influence ferulic acid esterase activity in kilned malt. In the case of acetic acid esterase, the influence of low pH of steeping water (5.2) on the degree of enzyme inactivation was evident; acetic acid esterase was also easily inactivated at 45°C during mashing, hence the role of this enzyme during mashing is considered to be marginal.

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WPŁYW pH I TEMPERATURY NA AKTYWNOŚĆ ESTERAZY KWASU FERULOWEGO I ACETYLOESTERAZY W CZASIE SŁODOWANIA I ZACIERANIA SŁODU

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Esteraza kwasu ferulowego i esteraza kwasu octowego są pomocniczymi enzymami biorącymi udział w degradacji arabinoksylanów w czasie słodowania ziarna i zacierania słodu. W pracy określono wpływ podwyższonej temperatury słodowania (zgodnie z technologią "Activated Germination Malting") oraz wpływ obniżenia pH wody użytej do namaczania ziarna jęczmienia na zmiany aktywności wymienionych enzymów. Obniżenie pH wody użytej do namaczania jęczmienia (z 7,4 do 5,2) nie miało wpływu na zmiany aktywności esterazy kwasu ferulowego w czasie słodowania, zaś podniesienie temperatury słodowania z 14°C do 22°C wywołało obniżenie aktywności wymienionego enzymu w gotowych słodach badanych odmian. Esteraza kwasu ferulowego nie była termostabilna i obniżenie aktywności enzymu zaobserwowano już w temperaturze 35°C. Dlatego też, w celu utrzymania aktywności esterazy kwasu ferulowego w czasie zacierania słodu, należy zastosować przerwę w temperaturze poniżej 40°C podczas początkowych etapów procesu.

Esteraza kwasu octowego jest kolejnym enzymem biorącym udział w degradacji arabinoksylanów w czasie słodowania ziarna i zacierania słodu. Namaczanie ziarna jęczmienia obu badanych odmian w wodzie o obniżonym pH (z 7,4 do 5,2) wywołało inaktywację esterazy kwasu octowego i w efekcie brak aktywności enzymu w gotowych słodach. Esteraza kwasu octowego uległa inaktywacji podczas zacierania w temperaturze 45°C i rolę tego enzymu w czasie degradacji arabinoksylanów zawartych w słodzie jęczmiennym można określić jako marginalną.